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PATHOPHYSIOLOGY OF ACUTE NONSPECIFIC DIARRHEA:

UPTAKE OF EXOTOXINS AND OTHER MACROMOLECULES AND THEIR EFFECT ON THE INTESTINE

ANNUAL REPORT

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ABSTRACT

Utilizing horseradish peroxidase (molecular weight 40,000) as a model tracer, we have been investigating macromolecular transport in the small intestine. Light and electron microscopy have shown that the tracer enters absorptive cells and after passing through a system of canalicular, vesicular, and vacuolar structures enters the intercellular space and lamina propria.

The relative capacity of jejunum and ileum to transmit intact macromolecules was evaluated through use of everted gut sacs incubated *in vitro* with their mucosal surface exposed to solutions of the tracer. These studies showed the model protein to be transmitted to a much greater extent by sacs prepared from jejunum as compared with ileum.

In vivo experiments following cannulation of the main mesenteric lymphatic vessel have shown that small but significant amounts of the tracer can be rapidly transmitted across the small bowel into its lymphatic efflux.

For the initial phases of our work on the permeability of the small intestine to macromolecules, we have focused on the use of horseradish peroxidase (a protein of 40,000 molecular weight) as a model tracer substance. We have begun our studies with this substance rather than a substance of greater intrinsic biological interest because of the following factors:

1. The peroxidase is readily available commercially in highly purified form at relatively modest expense.

2. It can be localized cytochemically at both the light microscopic and ultrastructural levels.

3. The enzyme can be quantitated in tissues and bodily fluids by means of an easy, rapid, sensitive, and accurate assay system.

It is our plan to extend the findings with this especially convenient protein tracer to more difficult to study macromolecules of pathophysiological interest such as enterotoxins, endotoxin of Gram negative bacteria, and vaccines and toxoids potentially useful for inducing local secretory immunoglobulin production within the lamina propria of the gut. The work with horseradish peroxidase and other readily demonstrable model tracer proteins ought to provide the leads necessary to identify the processes involved in transport of enteric disease-related macromolecules, including factors which can stimulate or suppress small intestinal absorption of such macromolecules as bacterial and parasitic toxins.

Our results so far fall into three general categories, all employing horseradish peroxidase as the model tracer.

1. Cytochemical localization

a. Light microscopy: In control experiments, the endogenous peroxidase activity of the small intestine was limited to erythrocytes and leucocytes of the lamina propria. In animals which had received peroxidase injected into

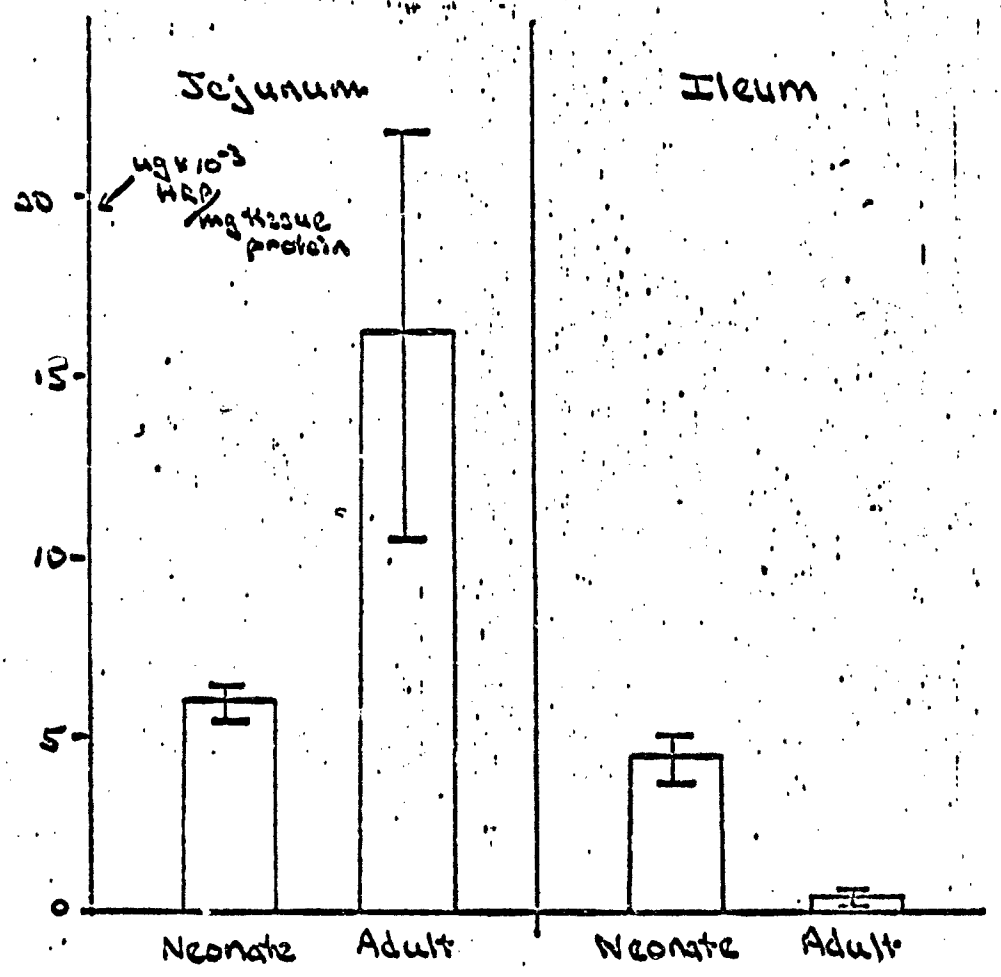
the lumen of ligated small bowel segments, there was deposition of reaction product along the surface of the absorptive cells in the area of the brush border. Within the absorptive cells themselves, peroxidase was found most prominently within small droplets of the apical cytoplasm, especially in the area immediately above the nucleus. The greatest number of peroxidase containing droplets occurred in the most mature cells at the tips of the villi, and considerably less activity was observed in cells along the sides and base of villi. The peroxidase was not present in the crypt epithelial cells. In contrast to the droplet-like deposition of the more normal absorptive cells, there was a diffuse distribution of peroxidase within sloughed epithelial cells of the lumen and within senescent cells in the process of extrusion from the tips of villi. Although the vast majority of goblet cells exhibited no peroxidase activity, occasional goblet cells did show considerable deposition of reaction product within them.

b. Electron microscopy: Even in those absorptive cells showing little or no peroxidase absorption, dense deposits of reaction product could frequently be seen to cover the external surface of microvilli, in the region of their glycocalyx. Peroxidase was not detected within the core of the microvilli but was distributed along the luminal surface of the intermicrovillous pits. Except for cells about to be extruded at the tips of villi, intracellular peroxidase was localized exclusively within membrane limited structures. In the apex of the cells, peroxidase was found within membrane lined vesicular and tubular structures, and deeper within the cell there was a general tendency for the tracer to be accumulated within larger and larger vacuolar structures surrounding the apical pole of the nucleus. Structures were commonly found suggesting that

peroxidase might have been passed within absorptive cells by either a process of fusion of vacuoles or transfer from tubular structures to vacuoles. The largest peroxidase containing vacuoles (ranging up to a micron or more in size) were concentrated within the zone immediately above the nucleus, often in close apposition to the Golgi apparatus. Reaction product was also located in some of the smooth vesicles intimately associated with typical stacks of Golgi cisternae. Although peroxidase could be readily localized in the intercellular spaces, the reaction product could not be found within the basal cytoplasm of absorptive cells. Therefore, it would appear that the peroxidase was transmitted to the extracellular space at about the level of the nucleus. Marked accumulations of raction product occurred in the narrow intercellular space between the complex interdigitations of neighboring cells and in the much wider dilatations of the intercellular space in the infranuclear zone. Extracellular reaction product was also seen surrounding lymphocytes in the process of transmigration through the mucosa (emperipolesis) and between the apical poles of absorptive cells and goblet cells when the latter was in the process of extruding mucus. The peroxidase was also present in the space between the basal lamina and the plasmalemma of the basal surface of absorptive cells, within the area of the basal lamina itself, and in the connective tissue space of the lamina propria.

These findings indicate that horseradish peroxidase, an enzyme of 40,000 molecular weight can be taken up be a characteristic membranous subcellular system within intestinal absorptive cells and ultimately is transmitted by these cells to the extracellular space of the lamina propria. Since the technique employed to demonstrate the peroxidase depends on the retention of its enzymatic function, the model tracer must be transmitted relatively intact.

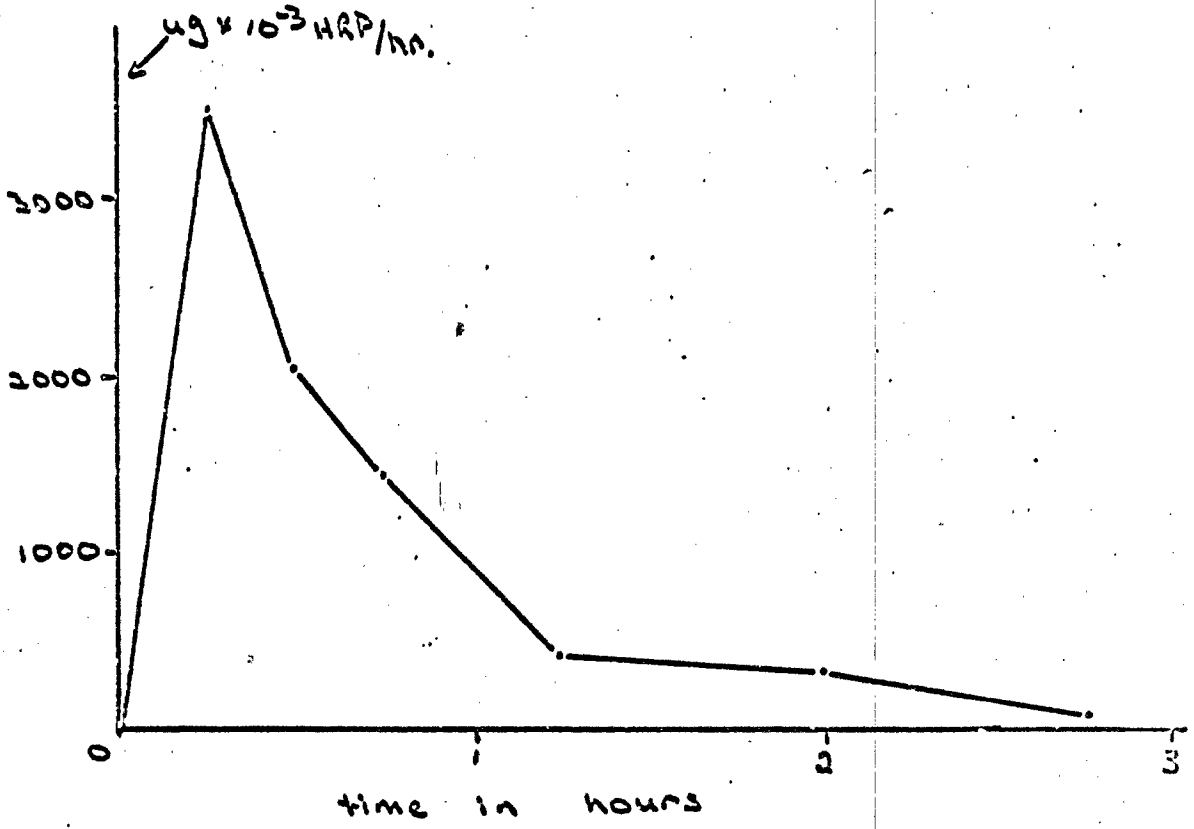
2. In vitro studies: Using the everted sac method of Wilson and Wiseman (J. Physiol., 123:116-125,1954) it has been possible to use small bowel segments for studying the transfer of horseradish peroxidase from the mucosal surface to serosal fluid. This method involves using small segments of intestine, turned inside out, filled with fluid, and tied at both ends. The peroxidase solution is then applied to the mucosal surface. The results of these studies have shown that everted sacs constructed from adult jejunum have a greater ability to transmit peroxidase than do sacs made from segments of adult ileum. The situation in the adult is in contrast to the neonatal gut, in which ileum and jejunum have shown equivalent facility for transporting the peroxidase. Not only does the adult jejunum have a greater facility for transmitting peroxidase than does the adult ileum but the capacity of the adult jejunum to transport peroxidase also exceeds the capacity of neonatal jejunum and ileum to transmit the enzyme. These results are summarized in graphic form on the following page.



In Vitro Absorption of Horseradish Peroxidase in Rats

3. In vivo studies employing lymphatic cannulations: In these studies the mesenteric lymphatic duct is cannulated under pentobarbital anesthesia, and at the same time a polyethylene catheter for infusion of a peroxidase containing solution is inserted into the jejunum. Following administration of the peroxidase, serial samples are taken of lymph fluid, and these samples are assayed for their peroxidase content. So far these studies have demonstrated that small, but significant amounts of horseradish peroxidase can be rapidly transmitted across the gut mucosa to the lymphatic efflux of the bowel. The results of a series of these experiments are tabulated below, and the following page contains a graph of a typical experiment in which good absorption of peroxidase occurred.

Time of expt. after lymphatic cannulation (hours)	Amount transmitted into lymph	Time of peak absorption (mins.)	Dose/kg. of body weight
3-4	593	90	20
3	4659	10	28
2-3	1112	15	29
3	267	15	21
3	70	45	5
16	2248	15	14
16	290	15	4.2
16	49	30	20
16	trace	90	20
16	trace	45	20



In Vivo Absorption of Horseradish Peroxidase in Adult Rats (16 hr. post op.)

SUMMARY

Preliminary to the study of the uptake and effect of exotoxins and other enteric macromolecules of pathophysiological importance, we have been conducting a series of experiments utilizing horseradish peroxidase as a model tracer. Since it is a large molecular weight substance (40,000 molecular weight) which can be studied by cytochemical and quantitative assay, this enzyme is ideally suited as a model substance for those studies.

Our methods of investigation and the results of our experiments to date can be summarized as follows:

1. Light and electron microscopy: horseradish peroxidase enters absorptive cells, and after passing through a membrane limited system of canalicular, vesicular, and vacuolar structures enters the intercellular space at or about the level of the nucleus. The protein then traverses the basement membrane to enter the intercellular space of the lamina propria.

2. In vitro: Everted gut sac experiments show that adult ileum and neonatal jejunum and ileum transmit peroxidase across their mucosa equally as well, but jejunum from mature animals has a significantly greater capacity than either neonatal gut or adult ileum for transmitting the tracer from the mucosal surface to serosal fluid.

3. Lymphatic cannulations: These in vivo experiments have shown that small but significant amounts of the model macromolecule are rapidly transmitted to the lymphatic efflux of the bowel.

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electron microscopy						